Introduction

Gene therapy is an attractive approach for treating inherited disorders. However, current techniques have not yielded many successful results in clinical trials. There are two primary gene delivery systems, viral and nonviral. Viral vectors such as retroviruses, lentiviruses, and adenoviruses have been proven to deliver genes efficiently; however, the possibility of negative outcomes resulting from viral transformation cannot be completely ruled out. In contrast, various types of nonviral vectors have been recently reported and are attracting considerable attention because they are easier to handle and induce weak immune responses.

Cationic polymers such as polyethylenimine (PEI) and poly(*N*,*N*-dimethylaminopropyl acrylamide) (PDMAPAAm), which can generate nanoparticles by forming polyion complexes, that is, "polyplexes" with DNA, are highly anticipated to be major carriers in nonviral gene delivery systems due to their many advantages compared to virus systems. However, the primary obstacle to implementing an effective gene therapy using cationic polymers remains their relatively inefficient gene transfection in vivo when compared with those of viral vectors.

To enhance gene transfection using cationic polymers, numerous studies have been performed by various approaches as follows: (1) chemical synthetic engineering, in which the kind and composition of the polymers are modified,[1,2] (2) biochemical, in which targeting ligands such as galactose, mannose, transferring, or antibodies are incorporated into the polymers,[3,4] and (3) functional molecular engineering, in which stimulus-response polymers with light- and thermal reactivity are designed as high performance vectors.[5−7] However, few studies have examined the molecular structure of cationic polymers, which are usually synthesized by conventional radical polymerization. Some exceptions include analysis of the effects of polymer chain length changes, polymer composition, and complex multibranching polymers. In these cases, structural analysis is impossible. Because achieving precise molecular design, including the molecular weight and three-dimensional structure, by conventional radical polymerization is quite difficult in general, the systematic structure-dependence of cationic polymers in gene transfection has not been established.

Iniferter-based living radical polymerization as a base for macromolecular architecture design of hyperbranched SVs will be considered here. Furthermore, we will describe four strategies for designing SV families using different approaches, which include (1) number of branches and precise chain lengths of branches; (2) biochemical modification of SVs by combination with targeting ligands; (3) cross-linking of SVs for the formation of further complex structures, namely, cross-linked SVs (CSVs); and (4) block copolymerization to form blocked SVs (BSVs). Finally, (5) a novel transfection method using thermoresponsive BSVs will be described.

Iniferter-Based Living Radical Polymerization

Photoliving polymerization can control polymer properties without catalysis and can be conducted under mild or extreme conditions using inexpensive instruments. This is in contrast to other precision polymerizations such as metal-catalyzed controlled radical polymerization, nitroxide-mediated controlled radical polymerization, and reversible addition−fragmentation chain transfer (RAFT) polymerization. One of the most popular photoliving polymerizations is iniferter (an iniferter acts as an initiator, transfer agent, and terminator)-based radical polymerization, which was pioneered by Otsu et al. in 1982.[8] *N*,*N*-Diethyldithiocarbamylmethylbenzene is a frequently used iniferter. The unique feature of this iniferter polymerization is that it proceeds in a controlled manner, in which "active" and "dormant" propagating chain ends are reversibly equilibrated under ultraviolet (UV) light irradiation. Using this polymerization, we successfully synthesized block copolymers having the ability to control the block chain lengths with minimal transfer or termination reactions.[9,10] Photoreactions are a suitable technology in the field of biomaterials because they can proceed without toxic catalysts. Therefore, iniferter-based polymerization was applied to modify naturally occurring polymers.[11] Furthermore, homo- or block-graft polymerized surfaces with regional precision were designed in order to create biocompatible surfaces.[12−15]

However, the standard iniferter polymerization method is not perfect with respect to conversion and termination. Most studies over the years have employed UV light sources, which frequently induce irreversible termination because of the exposure to excessive high-energy wavelengths. Therefore, we determined the optimal irradiation wavelength for precise polymer design using monochromatic light in the range from 330 to 400 nm at approximately 10 nm intervals using the tetrafunctional iniferter 1,2,4,5-tetrakis(*N*,*N*-diethyldithiocarbamylmethyl)benzene in the presence of *N*,*N*-dimethylaminopropyl acrylamide (DMAPAAm).[16] Polymerization was maximum at 370 nm and proceeded in a controlled manner up to approximately 30% conversion and up to a molecular weight of approximately 40 000 with a relatively limited polydispersity index (PDI: approximately 1.6).

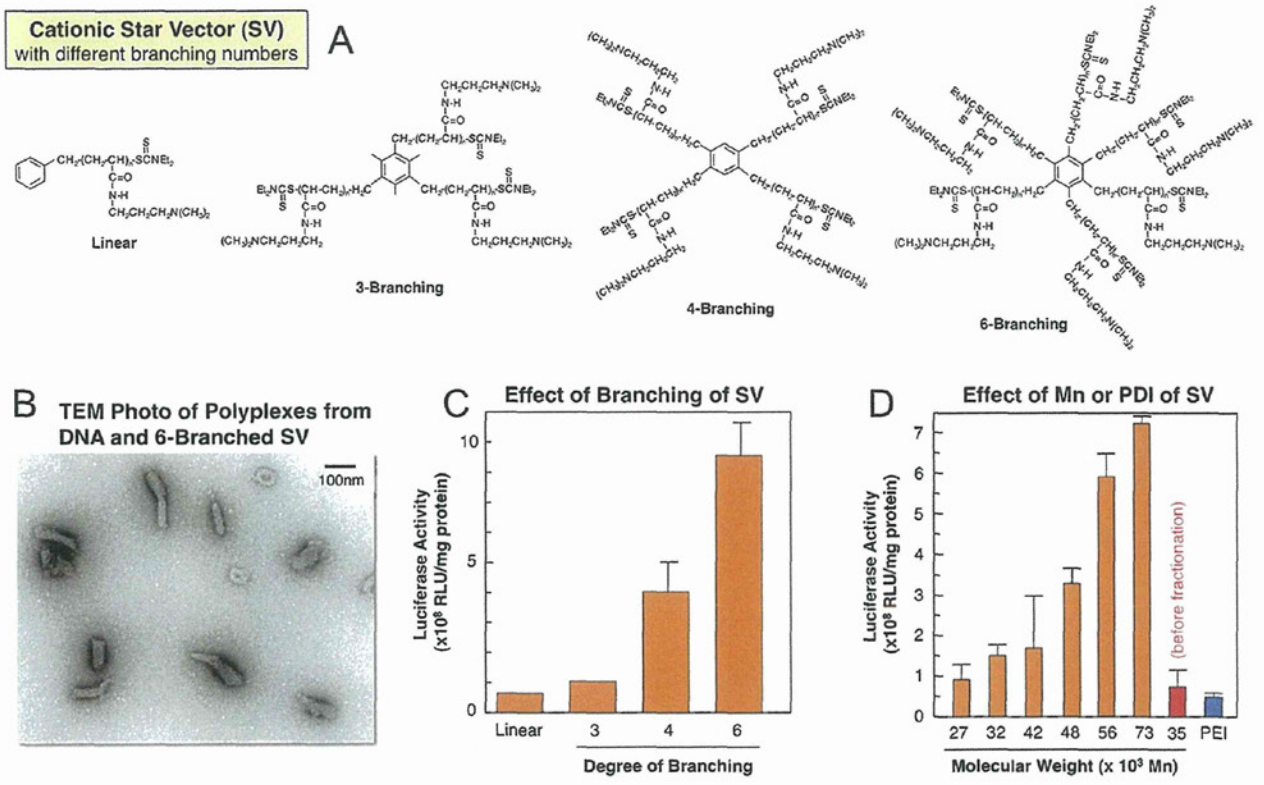

FIGURE 1. (A) Chemical structures of SVs with different branching numbers, which were synthesized by iniferter-based photoliving radical polymerization of 3-(*N*,*N*-dimethylamino)propylacrylamide (PDMAPAAm) from the respective multifunctional iniferters, *N*,*N*-diethyldithiocarbamate-derivatized benzenes. (B) Transmission electron microscopy (TEM) image of a polyplex from DNA (pGL3-control) and 6-branched SV of PDMAPAAm (M_n: 18 000, PDI: 1.4). (C) The transfection activity of the polyplexes prepared from DNA (pGL3-control) and 6-branched SVs (M_n: 18 000, PDI: 1.4) with varying degrees of branching in COS-1 cells. (D) The transfection activity of the polyplexes prepared from DNA and the 6 fractionated 4-branched SVs of PDMAPAAm (M_n: 27 000–73 000) with narrow PDI (1.1–1.2) or linear PEI in COS-1 cells.

The theoretical number of *N*,*N*-diethyldithiocarbamate functional groups after polymerization was obtained. Thus, this simple optimization procedure improved the reliability of the iniferter method.

Approach 1: Effects of Branching Number, Molecular Weight, and Polydispersity of SVs

We attempted to improve the utility of star-shaped, hyperbranched cationic polymers as a base chemical structure for a novel high-performance gene carrier. SVs were prepared by iniferter-based living radical polymerization of DMAPAAm using the respective multidithiocarbamate-derivatized benzenes (multifunctional iniferters). As the first approach, we synthesized a series of linear and branched (3, 4, or 6) cationic polymers, with the same approximate molecular weight (M_n: 18 000) and PDI (1.4) (Figure 1A).[17] The PDI was determined by GPC using a high-performance liquid chromatography system, calibrated for molecular weight with narrow weight distribution poly(ethylene glycol) standards. All polymers produced polyion complex (polyplexes) when mixed with a luciferase-encoding plasmid DNA (pGL3-control plasmid). The size of the polyplexes, determined by dynamic light scattering (DLS), ranged from approximately 150 nm in diameter. Similar value was observed by TEM (Figure 1B). Higher gene expression was obtained with increased branching accompanied by little cytotoxicity. Gene expression relative to the linear polymer was about 2, 5, and 10 times higher for the 3-, 4-, and 6-branched polymers, respectively (Figure 1C). The precise change in polymer branching permitted the control of transfection activity. As the degree of branching increases, the density of cationic charges in the branched polymers increases, which may result in high compaction of DNA polyplexes to achieve high gene expression levels.

The gene transfection efficiency of cationic polymers strongly depends on their structures as demonstrated by the branching number. Similarly, molecular weight, which is one of the main structural parameters, exerts the greatest influence on gene transfection efficiency and has been evaluated by many researchers.[18–20] Synthetic polymers

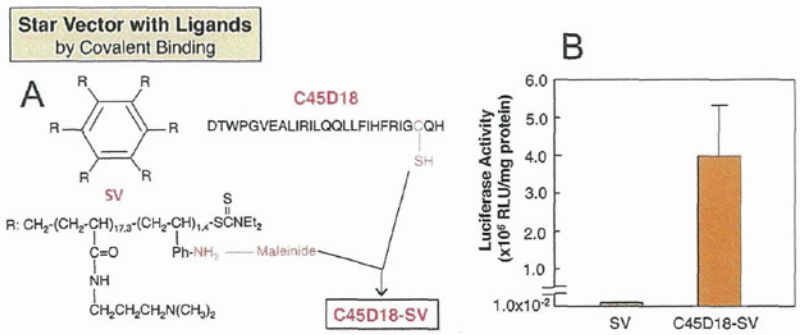

FIGURE 2. (A) Chemical structure of 6-branched SVs of PDMAPAAm (Mn: 18300, PDI: 1.4) derivatized with C45D18 peptide (C45D18-SV). (B) Gene transduction into human macrophages. Monocyte-derived macrophages were prepared from healthy humans by culturing peripheral blood mononuclear cells for 6 days in the presence of 100 ng/mL macrophage colony-stimulating factor (M-CSF). Then, the cells were deprived of M-CSF for 4–5 days and subjected to experiments.

invariably have heterogeneous molecular weights even if they are synthesized by living radical polymerization. Unfortunately, molecular weight distribution, including PDI, has not been necessarily described in reports in this field. Using uniform polymers with monodispersity is ideal for precisely evaluating transfection efficiency.

To examine precisely the effect of molecular weight of the SV in terms of gene transfection efficiency, we separated 4-branched SVs of PDMAPAAm (M_n: 35 000, PDI: 1.6) into several different molecular weight fractions with low PDI ranging from 1.1 to 1.2 by using size-exclusion chromatography.[21] The highest luciferase transfection activity was obtained for the SVs with the highest molecular weight (73 000) and was more than 7 times greater than that for the SVs with the lowest molecular weight (27 000), the nonfractionated SVs or PEI (ExGen500), which is one of the major commercially available typical polymeric vectors used as a positive control (Figure 1D). Transfection activity correlated more with SV amphiphilicity or hydrophobicity, surface potential, and condensate density of the polyplexes than with particle size. It was very difficult to clearly explain the relationship between the transfection activity and the physicochemical properties of SVs or the polyplexes, such as critical micellar concentration of the SV, mean diameter, ζ-potential of the polyplexes, and pDNA condensation potential of the polyplexes. However, SVs with the highest molecular weight and activity could be easily isolated by using size-exclusion chromatography. In comparison with fractionated SVs with narrow molecular weight distribution, slight differences in molecular weight resulted in considerable differences in the physicochemical properties and transfection efficiencies. We consider that the optimal molecular weight is different for each polymeric vector species because the vectors exhibit different physicochemical properties. Therefore, precise and reliable evaluation of transfection activity must be performed using polymeric vectors with narrow molecular weight distributions. Size fractionation will also be useful for enhancing the gene transfection activity of other cationic polymers.

Approach 2: Combination with Ligands

Vectors that express ligands are effective in delivering a target to specific cells. Vpr, a human immunodeficiency virus type-1 nonstructural gene product,[22] is thought to transport viral DNA from the cytoplasm to the nucleus in resting macrophages.[23] A peptide encompassing amino acids 52–78 of Vpr (C45D18) promotes nuclear trafficking when conjugated to recombinant proteins.[24] Gene expression in resting macrophages is facilitated when C45D18 is conjugated to 6-branched SVs of PDMAPAAm (M_n: 18 300, PDI: 1.4) terminated with oligo(4-aminostyrene) (1.4 of 4-aminostyrene units in average were terminated for each branch) (Figure 2A).[25] Although there was no difference between SV alonse and C45D18-SV with respect to gene transfer into growing cells, C45D18-SV resulted in more than 40-fold greater expression of the exogenous gene when transfected into chemically differentiated macrophages and quiescent monocyte-derived human macrophages (Figure 2B). These data suggest that C45D18 contributes to improving the ability of a nonviral vector to transduce macrophages with exogenous genes. We postulate that the C45D18-SV system will be effective for transfecting other types of resting cells.

In contrast, the tripeptide sequence arginine (Arg)-glycine (Gly)-aspartic acid (Asp) (RGD) found in the active site of vitronectin binds to integrin $\alpha_V\beta_3$ and almost half of the other 22 known integrins.[26] RGD peptides are exploited by pathogenic microorganisms, such as the foot-and-mouth disease virus, for cell entry.[27] A great advantage of integrin targeting is that internalization occurs by a "zippering"

mechanism that allows the uptake of relatively large structures such as bacteria. Therefore, several cationic polymer vectors combined with RGD peptides were developed for tumor-targeted gene delivery.[28] For example, RGD-containing peptides were coupled with PEI with or without a PEG spacer. The RGD-modified PEI showed a significant increase in transfection efficiency as compared with only PEI in endothelial cells. Almost all RGD-mediated gene delivery systems have been described with the chemical derivatization of RGD peptides to vector compounds.

We investigated the feasibility of using SV in endothelial cells for enhancing gene transfection by adding the RGD peptide (Figure 3A).[29] We strongly believed that if the coating of the polyplex surface with the RGD peptides occurs only by the addition of these peptides, then the gene transfection efficiency for endothelial cells would be enhanced, similar to that in the case of chemical derivatization of the RGD peptides. The addition of the RGD-containing peptide (GRGDNP) to the solution of polyplex from 4-branched SVs of PDMAPAAm (M_n: 18 000, PDI: 1.4) and the luciferase-encoding plasmid DNA led to a marked inhibition of polyplex aggregation, indicating the coating of the polyplex surface with RGD peptides (Figure 3B). A transfection study on endothelial cells showed that luciferase activity increased with the amount of RGD peptides added to the polyplexes (Figure 3C). The activity further increased 8-fold compared to that without RGD addition when cyclic RGD peptides (RGDFV) were used. In both cases, the surplus RGD peptides might bind to the integrin receptor and prevent binding with RGD peptide, thereby coating the polyplexes. Gene delivery to endothelial cells was significantly enhanced by the addition of only RGD peptides to SV-based polyplexes. The enhancement of gene transfer by the addition of the RGD peptide may be applied to other cationic polymer vectors as well because the RGD peptide could coat the cationic surface of the polyplexes. The surface-coating ability may be enhanced by the introduction of anionic amino acids such as aspartic acid or glutamic acid into the RGD peptide.

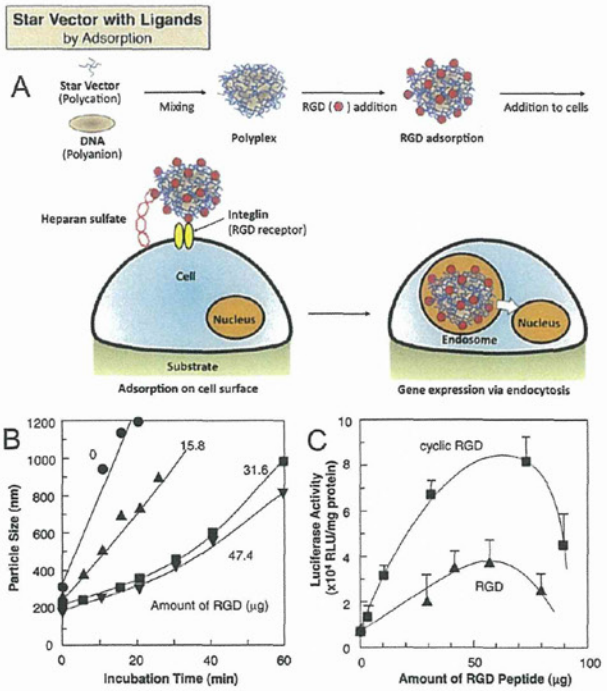

FIGURE 3. (A) Illustration of the method of gene transfection to endothelial cells by using 4-branched SVs of PDMAPAAm with the addition of the RGD peptide. (B) Incubation time-dependent changes in the cumulant diameter of the polyplexes from DNA (pGL3-control) and 4-branched SVs (M_n: 18 000, PDI: 1.4) in aqueous solutions in the presence of RGD peptides (GRGDNP). (C) Transfection of the same polyplexes in the presence of RGD peptides (linear GRGDNP or cyclic RGDFV) to endothelial cells.

Approach 3: Intermolecular Cross-Linking

In the first approach, the greater the number of branches, the higher is the efficiency of gene expression. Because additional complex branching by cross-linking SVs was expected to further improve gene transfection efficiency, we attempted a photocross-linking strategy using macroiniferters.[30] As demonstrated in the iniferter-based living radical polymerization section, polymerization due to an iniferter occurs in the presence of a monomer. However, in the absence of monomers, chain transfer of the generated benzyl radicals and dithiocarbamyl radicals to the solvent or polymer matrix results in simultaneous coupling between the radicals. Coupling between benzyl radicals and dithiocarbamyl radicals is reversible, and these radicals are regenerated by UV reirradiation. Coupling between dithiocarbamyl radicals generates *N,N,N′,N′*-tetraethylthiuram disulfide, which is dissociated to dithiocarbamyl radicals by UV irradiation because the coupling is also reversible. In contrast, coupling between benzyl radicals generates bibenzyl, which is not dissociated by UV irradiation. Therefore, benzyl radicals are selectively consumed by UV irradiation to generate bibenzyl. As expected, a dimer was produced from a PEG derivative with dithiocarbamate at one terminus, and a polymer was produced from the PEG derivative with dithiocarbamate at both termini.[31] This study indicated that the terminal ends of the dithiocarbamate-derivatized polymers could be cross-linked by photoirradiation alone, without using a chemical cross-linking agent such as glutaraldehyde or diisocyanate. The cross-linking may be applied to molecular architecture to prepare more complex-shaped polymers, such as mesh or hyper-branch structures.

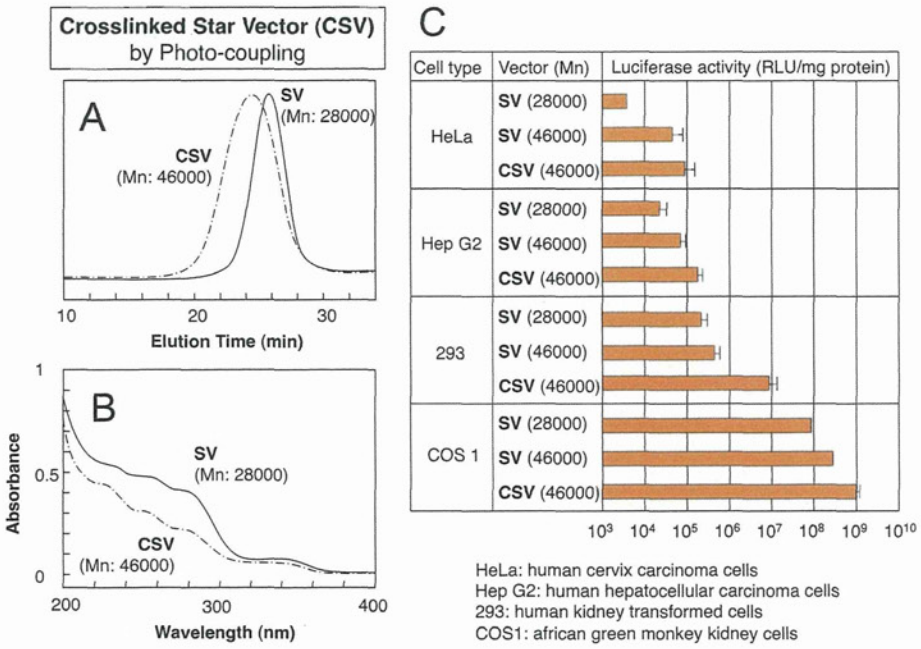

FIGURE 4. (A) GPC curves and (B) UV adsorption spectral changes after photoirradiation of 4-branched SVs of PDMAPAAm (M_n: 28 000, PDI: 1.4) to obtain CSVs (M_n: 46 000, PDI: 2.0) (irradiation time, 240 min). (C) Transfection of four different cell lines using polyplexes prepared by mixing the DNA (pGL3-control) and the SV or CSV.

We used 6-branched SVs of PDMAPAAm as a precursor for photocross-linking. With increasing irradiation time, the molecular weight of the CSVs increased through terminal coupling of the SVs (Figure 4A); this effect was confirmed by a decrease in the absorbance of dithiocarbamate (Figure 4B).[30] As expected, cross-linking the SVs the gene transfection efficiency increased dramatically in several types of cells (Figure 4C). The luciferase activity with CSVs (M_n: 46 000, PDI: 2.0) was at least 1 order of magnitude in the original SV (M_n: 28 000, PDI: 1.4). The activity of CSVs was at least 2-fold higher than that of non-CSVs of the same molecular weight (M_n: 46 000, PDI: 1.4). Designing nano-architectural macromolecular polymeric DNA carriers by hyper-branching or cross-linking or other methods has been considered an important strategy for improving the transfection efficiency. Therefore, we consider that SV cross-linking, which can induce an increase in the cationic charge density, could be a key strategy for enhancing the transfection efficiency of cationic polymers.

Approach 4: Blocking of SVs

Nonionic Blocking. The conjugation of cationic polymers with a hydrophilic and biocompatible polymer such as polyethylene glycol (PEG)[32,33] provides a major vector-modification strategy to improve transfection efficiency.[34,35] Surface modification of these PEG conjugates increases the DNA delivery system's half-life in blood circulation. Therefore, linking the nonionic hydrophilic chains with SV DNA condensates was expected to improve gene transfection efficiency by shielding the particles from nonspecific interactions and conferring greater stability upon them by a process similar to PEGylation of polyplexes.

As the fourth approach, we designed polymers consisting of cationic and nonionic chains as inner and outer domains, respectively, by iniferter-based block radial polymerization (Figure 5A).[36] We synthesized 4-branched PDMAPAAm-poly(*N,N*-dimethylacrylamide) (PDMAAm)-blocked copolymers with copolymer compositions (unit ratio of DMAAm to DMAPAAm) ranging from 0.18 to 1.0 for 4-branched PDMAPAAm with molecular weights ranging from 20 000 to 50 000, and a PDI of 1.3–1.4. The polyplexes formed by block copolymers with molecular weights of 50 000 in the case of PDMAPAAm branches and 33 000 in the case of PDMAAm branches supported the highest luciferase activity, which was approximately 5-fold higher than that achieved with nonblocked SVs (Figure 5B). PDMAPAAm-PDMAAm-blocked star-shaped polymers (blocked star vectors [BSVs]) are an attractive novel class of nonviral gene delivery systems.

Interestingly, because the polyplexes were very stable in aqueous media even at 1 month after their formation, they

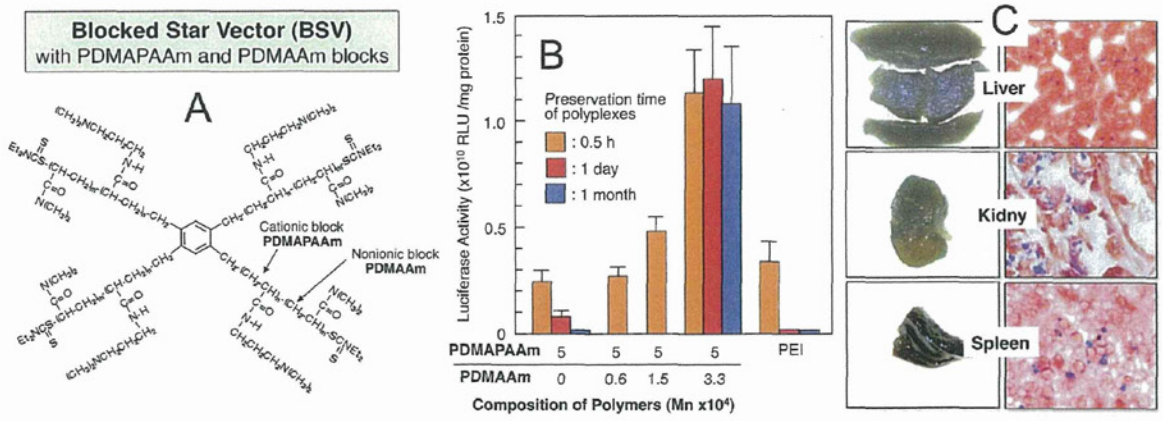

FIGURE 5. (A) Chemical structure of 4-branched BSVs containing an inner domain of cationic PDMAPAAm chains and an outer domain of nonionic PDMAAm chains. (B) Transfection efficiency of polyplexes prepared from DNA (pGL3-control) and the 4-branched BSV at different preservation times after preparation. For comparison, transfection efficiency data from PEI (M_n: 18 000) is also provided. (C) Expression of β-galactosidase in mouse organs (liver, kidney, and spleen) 48 h after injection of the polyplexes from DNA (*lac Z* gene, *pcDNA3.1/His B/lac Z)* and the 4-branched BSV with PDMAPAAm block (M_n: 50 000) and PDMAAm block (M_n: 33 000) from jugular vein. (left side) Macroscopic photos after staining with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). (right side) Histological sections further stained with eosin.

continued to show high activity, whereas nonblocked SVs showed approximately 60% loss of the activity after 1 day (Figure 5B). In the case of PEI, more than 90% of the activity was lost within 1 day after polyplex formation.

Furthermore, in preliminary in vivo studies conducted over 48 h, mice injected with the polyplexes containing the β-galactosidase-expression gene showed high levels of gene expression in the liver, kidneys, or spleen, without any detectable tissue damage (Figure 5C).[37] Upon staining with X-gal, these resected tissues showed complete green coloration because of the enzymatic decomposition of X-gal by the produced β-galactosidase. In addition, many blue spots of 5,5′-dibromo-4,4′-dichloro-indigo, produced by the decomposition, were clearly observed. These results suggest that BSV has potential for clinical use as a nonviral vector.

siRNA Delivery by BSV. RNA interference (RNAi) is the process by which double-stranded RNA (dsRNA) directs the sequence-specific degradation of complementary mRNA.[38,39] It is therefore an effective method for gene-function analysis as well as a potentially powerful therapeutic modality for silencing pathogenic gene products associated with cancer, viral infections, and autoimmune disorders. Small interfering RNAs (siRNAs) can be directly introduced into cells as synthetic linear siRNAs or short hairpin RNAs (shRNAs).[40] RNA polymerase III driven expression cassettes can be used to constitutively express shRNA molecules. Although both viral and nonviral vectors can be used to deliver siRNA into cells, viral vectors are not sufficiently capable of delivering siRNA-expressing constructs such as shRNAs. Commercially available cationic lipids such as Oligofectamine can efficiently deliver siRNAs into cells;[41] however, such cationic lipids are highly toxic and hence cannot be effectively used for systemic delivery of siRNAs in vivo.

We developed a novel nonviral gene silencing system using siRNA or shRNA complexes and BSVs with 4-branched PDMAPAAm (M_n: 50 000) and PDMAAm (M_n: 33 000) blocks (PDI: 1.4).[42] BSV is shown to condense and interact with siRNAs to yield stable BSV/siRNA polyion complexes, approximately 90 μm in diameter. Using these complexes, siRNA was successfully delivered to almost all human hepatocellular carcinoma cells, as confirmed by fluorescence microscopic examination, which showed Cy3 labeling in almost cells (Figure 6A). siRNAs could induce significant gene silencing in these cells without affecting cell viability (Figure 6B). We selected the lamin gene as the target for siRNA-mediated gene silencing. Lamin siRNA was used to assess the correlation of fluorescence with transfection efficiency and target gene silencing. Moreover, lamin A/C is abundantly expressed in most human, mouse, and rat cells, and knockdown of lamin mRNA does not affect cell viability. The gene-silencing efficacy of the BSV/siRNA polyion complexes was similar to that of a commercially available high-efficiency siRNA transfection reagent (Dharmafect 4; TR).

After injecting BSV/siRNA complexes into mice, effective gene silencing was observed in the liver and lungs (Figure 6C), indicating that these complexes were stable in vivo and retained their transfection efficiency after

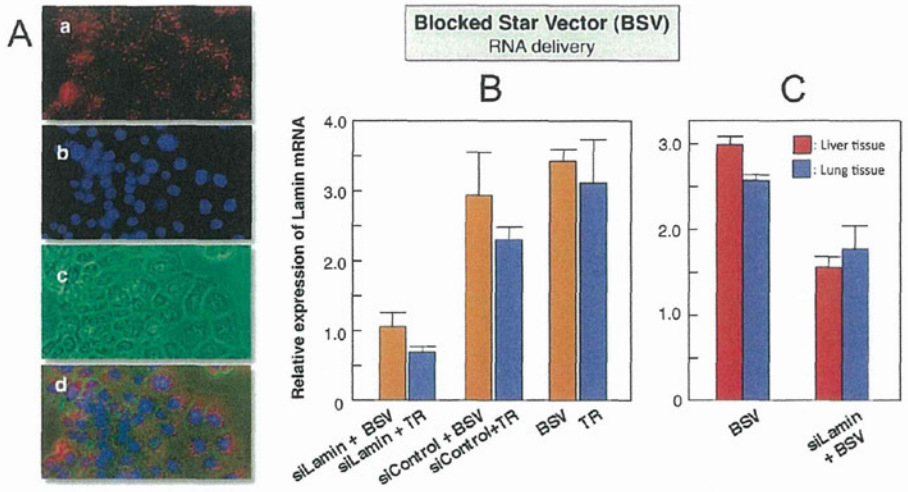

FIGURE 6. (A) Fluorescence microscopic image of Cy3-labeled siLamin (red color, siGL2 Lamin A/C siRNA, sense: 5′-GGUGGUGACGACGAUCUGGGCUdTdT-3′; antisense: 5′-AGCCCAGAUCGUCACCACCdTdT-3′) in human hepatocellular carcinoma cells delivered by polyplexes from the siRNA and 4-branched BSV with PDMAPAAm block (M_n: 50 000) and PDMAAm block (M_n: 33 000) (a), after staining the nucleus with diaminophenyl indole (DAPI; blue) (b), phase-contrast microscopic image for the observation of cell structure (c), and all three images merged into one (d). (B) In vitro lamin mRNA silencing efficiency of the polyplexes in human hepatocellular carcinoma cells, as analyzed by quantitative RT-PCR. siControl is designed to minimize the potential for targeting any known human or mouse genes (sense, 5′-UAGCGACUAAACACAUCAAUU-3′; antisense, 5′-UUGAUGUGUUUAGUCGCUAUU-3′). (C) In vivo lamin mRNA silencing efficiency of the polyplexes in mice liver or lung tissues, as analyzed by quantitative RT-PCR. The polyplexes were transfected by tail vein injection.

intravenous administration. Thus, BSVs can serve as carriers for siRNA and shRNA delivery both in vitro and in vivo and may thus offer a new approach to gene therapy.

Deposition Transfection by BSV. A reverse transfection method was developed as another practical approach for gene delivery.[43] This method is performed by culturing cells on a plasmid DNA-loaded substrate. DNA-loaded substrates are generally prepared by mixing DNA with cationic polymers that have been physically adsorbed or chemically bonded to the substrate. The cells were in direct contact with the DNA-loaded surface during the culture period; this differs from conventional transfection culture in which the contact time is limited. Furthermore, transfectional microarrays that permit parallel transfer of multiple genes into cultured cells were developed for high-throughput reverse genetics research because the reverse transfection method can be performed with spatial and temporal control.[44] However, it cannot be said that the transfection efficiency of this method is as good as that of the conventional transfection method. Therefore, an additional physical stimulus, such as an electric pulse, was used to enhance the transfection efficiency.

We designed a unique gene-adsorbent material possessing thermoresponsive properties in order to improve the reverse transfection method.[45] The SVs had 4-branched PDMAPAAm (M_n: 3000, PDI: 1.3) for binding to a plasmid DNA to form polyplexes and were blocked with poly(*N*-isopropylacrylamide) (PNIPAM) chains (M_n: 6000, PDI: 1.4) for surface deposition on a hydrophobic substrate (Figure 7A). PNIPAM is one of the most well-known thermoresponsive polymers.[46] Therefore, the SV was precipitated at approximately 35 °C owing to hydrophilic to hydrophobic conversion of the thermoresponsive polymer chains (Figure 7C). In ordinary reverse transfection, it is necessary to coat DNA on the culture substrate with cationic matrix materials to ensure that the DNA is firmly impregnated in the matrix that is adhered or bonded to the substrate. In contrast, in our method, the DNA was precipitated onto a culture substrate using a thermoresponsive polymeric adsorbent material immediately before the cells were seeded (Figure 7B). Further, in contrast to the conventional transfection and reverse transfection methods, our method allowed transfection experiments to be performed without the need for preculturing cells or precoating genes, respectively, at least 1 day in advance. Furthermore, serum-containing culture medium could be used.

When COS-1 cells were cultured on the polyplex-coated substrate in a culture medium, the luciferase activity was higher than that observed when (1) a DNA-coated substrate was used with or without SVs, before and after complete adhesion, or (2) a conventional transfection solution containing the polyplexes was used. With the proposed method, the luciferase activity was enhanced with an increase in the charge ratio and with

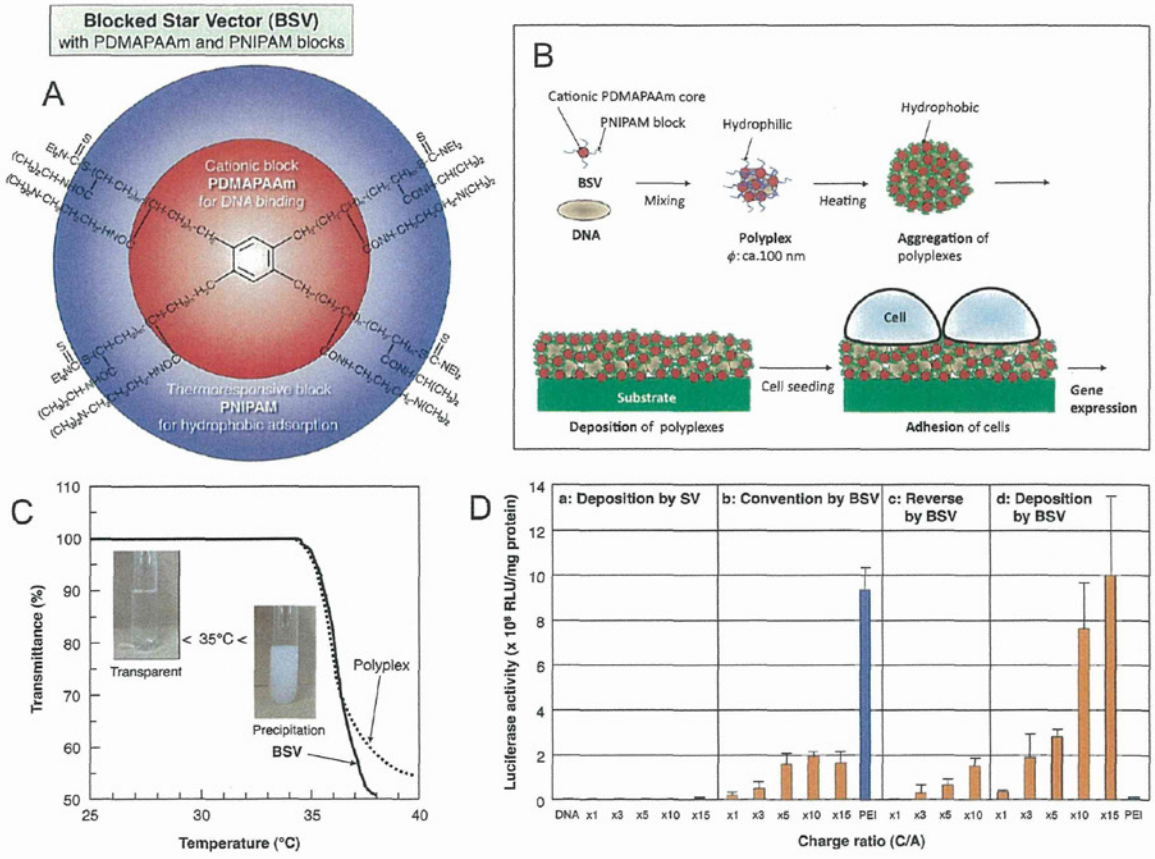

FIGURE 7. (A) Chemical structure of the 4-branched cationic thermoresponsive BSVs with PDMAPAAm (M_n: 3000, PDI: 1.4) and PNIPAM (M_n: 6000, PDI: 1.4) blocks as an adsorbent material for the deposition transfection method. (B) Illustration of the deposition transfection method. (C) Thermoresponsive changes in the transmittance of the BSV and its polyplex with DNA (pGL3-control). BSV concentration: 10 mg·mL^{-1}, DNA concentration: 5 mg·mL^{-1}. Heating rate: 0.5 °C·min^{-1}. (D) Transfection activity using the 4-branched SV of PDMAPAAm (M_n: 3000) (a) and the BSV in (b) the conventional solution method, (b) the reverse transfection method, and (d) the deposition method under different CA ratio.

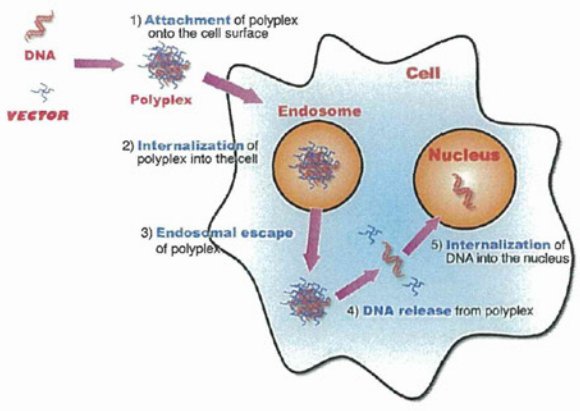

FIGURE 8. Estimated DNA delivery mechanism by cationic polymers including SV.

permissible levels of cellular cytotoxicity (Figure 7D). This novel transfection method, which is a modified reverse transfection method, is termed the deposition transfection method.

Interestingly, thermoresponsive SVs can bind to other anionic compounds.[47] When heparin was mixed with the thermoresponsive SVs, the resultant hydrophobic aggregates could be stably adsorbed onto the surfaces of several polymeric materials for antithrombogenic coating. Recently, by taking advantage of the cationic and thermoresponsive characteristics of PDMAEMA, a novel heparin bioconjugate with SVs was developed for highly effective heparin surface immobilization, based on a strategy similar to that described above.[48] In addition, PDMAEMA-based SVs could be applied as a novel agent for deposition transfection, where high and sustaine transgene expression was achieved by using small amounts of PDMAEMA.[49]

Conclusion

Four approaches for effective DNA or siRNA delivery based on optimizing the macromolecular design by using the

photochemistry of iniferters were described. All strategies improved the transfection activity. However, it was very difficult to clearly explain the relationship between transfection activity and physicochemical properties, such as amphiphilicity or hydrophobicity, particle size, surface potential, and condensate density of the polyplexes. DNA expression through the polyplexes probably occurs via five sequential steps as shown in Figure 8: (1) attachment of the polyplex onto the cell surface, (2) internalization of the polyplex into the cell, (3) endosomal escape of the polyplex, (4) DNA release from the polyplex, and (5) internalization of DNA into the nucleus. Virus are naturally equipped with unique mechanisms which they overcome obstacles at each step. Therefore, virus vectors such as retroviruses, lentiviruses, and adenoviruses have proven to be efficient means for gene delivery; however, the possibility of negative outcomes resulting from viral transformations cannot be completely ruled out. On the other hand, cationic polymers used for nonviral vectors have little physiological properties for overcoming the above-mentioned obstacles. Therefore, DNA transfection is induced by the progression of the obstacles at the sequential steps depending the physiochemical properties of the polymers or the polyplexes. However, despite our improved understanding of nonviral gene delivery systems, many questions related the underlying mechanisms remain unanswered. Any single physicochemical property of the system can have a positive or negative influence at each step. Therefore, an optimum balance in the properties involved is very important. Once a system for evaluating each stage of the transfection process has been established, the combination of properties required will become apparent, and this in turn will lead to the development of an ideal polymeric vector. At present, we believe that at least the following macromolecular properties are essential for structural designing useful polymeric vectors: (1) high molecular weight, (2) high degree of branching or complexity, and (3) narrow polydispersity.

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BIOGRAPHICAL INFORMATION

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FOOTNOTES

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Deposition Gene Transfection Using Bioconjugates of DNA and Thermoresponsive Cationic Homopolymer

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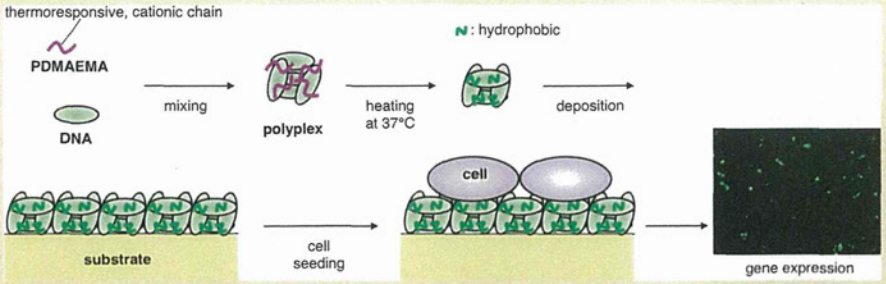

ABSTRACT: A poly(*N*,*N*-dimethylaminoethylmethacrylate) (PDMAEMA) homopolymer with both thermoresponsive and cationic characteristics was applied to a vector for use in deposition transfection. PDMAEMA with a molecular weight of 2.5×10^5 g mol^{-1} was synthesized by photoinduced radical polymerization. Polyplexes approximately 750 nm in size were formed by mixing PDMAEMA with luciferase-encoding plasmid DNA. The polyplexes had a lower critical solution temperature (LCST) of approximately 30 °C. In addition, they exhibited excellent adsorption and durability on a polystyrene surface, as confirmed by a surface chemical compositional analysis. When HeLa cells and primary cells were cultured on a substrate coated with the polyplexes, high transgene expression and cell viability of more than 90% were obtained at low charge ratios (PDMAEMA/plasmid DNA ratio) ranging from 2 to 8. In addition, transgene expression was sustained for over 2 weeks post-transfection whereas decreased expression was observed 5 days post-transfection when the conventional solution-mediated transfection method was used. Thus, high and sustained transgene expression as well as high cell viability can be realized by using small amounts of PDMAEMA as a deposition transfection material.

INTRODUCTION

Controlled gene delivery, e.g., by transfection, using biomaterial surfaces (substrate-mediated transfection) and transfection of specific tissues or cells (targeted transfection) are important in fields such as regenerative medicine and gene therapy.[1,2] In general, viral transfection is highly efficient, but it has several drawbacks. These include the potential for inducing acute inflammatory responses, integrating viral genes into the host genome, and limiting the DNA loading capacity.[3,4] Moreover, the use of viral carriers for controlled transfection is technically difficult because they cannot be readily modified. Cationic liposomes and polymers that can be used to generate gene-deliverable nanoparticles by forming polyion complexes (polyplexes) with plasmid DNA (pDNA) have been developed as nonviral carriers.[5,6] The transfection efficiency of nonviral carriers is lower than that of viral carriers; however, for controlled transfection, it is easier to introduce functional molecules such as signal peptides,[7,8] bioactive molecules,[9,10] thermoresponsive molecules,[11,12] or pH-responsive molecules[13,14] into nonviral carriers than viral carriers.

In a previous study, we designed a surfactant polymer for the thermoresponsive surface immobilization of pDNA.[15] The polymer comprised four AB-type block branches, each incorporating two different chemicals: a cationic poly(*N*,*N*-dimethylaminopropylacrylamide) (PDMAPAAm) block and a thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) block (PDMAPAAm-PNIPAM). PNIPAM is the most popular thermally sensitive water-soluble nonionic polymer available and has a lower critical solution temperature (LCST) of approximately 32 °C. As shown in Figure 1A, the tertiary branched PDMAPAAm-PNIPAM block copolymer formed polyplexes with pDNA and was deposited on a cell culture surface at 37 °C. High transgene expression was achieved by using the cells seeded on to the polyplex culture surface. This surface-mediated transfection method is referred to as deposition transfection.

PDMAEMA is a cationic polymer and is used widely as a nonviral gene delivery carrier.[16−18] On the other hand, it is also a thermoresponsive polymer that has LCST, ranging from 34 to 37 °C.[19−21] However, little biomedical research has been carried out on the dual characteristics of this polymer. Recently, we developed a novel aqueous anti-thrombogenic coating

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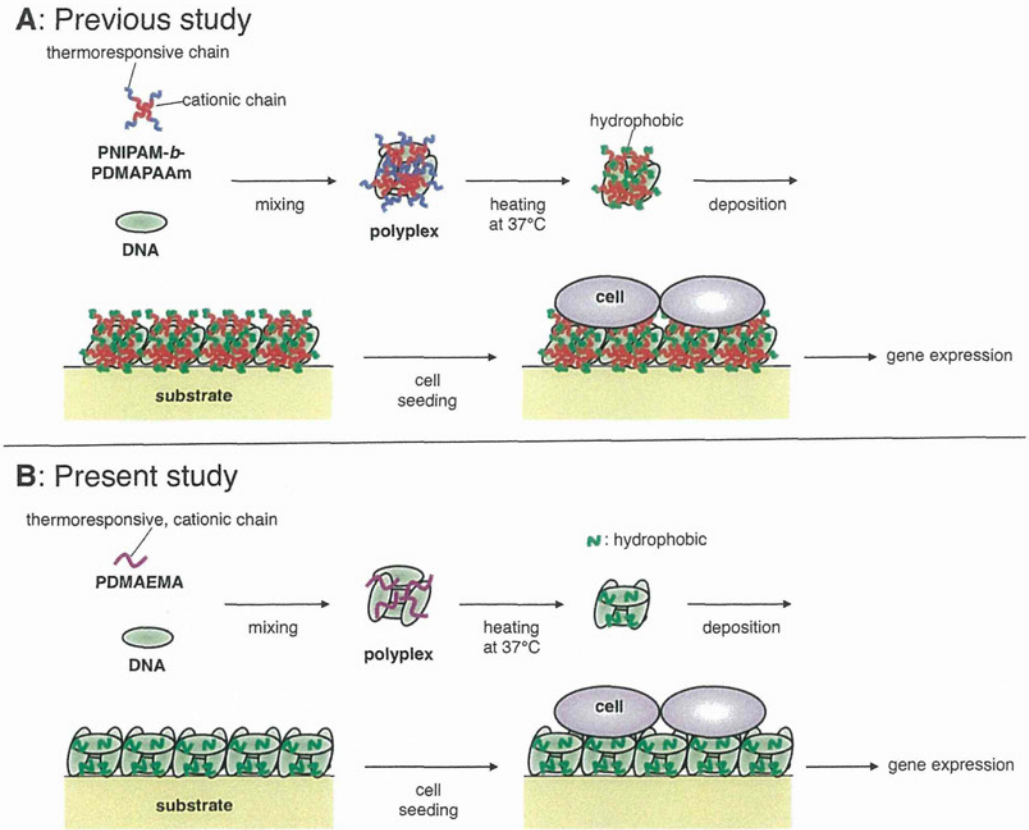

Figure 1. Deposition transfection method using (A) the tertiary branched thermoresponsive cationic block copolymer (PNIPAM-b-PDMAPAAm) in our previous study[15] and (B) thermoresponsive cationic homopolymer (PDMAEMA) in this study.

material that combines the thermoresponsive and cationic characteristics of PDMAEMA and can effectively immobilize heparin/PDMAEMA bioconjugates onto biomaterial surfaces at physiological temperatures.[22] By using PDMAEMA, the amount of polymer necessary to produce the heparin bioconjugate was reduced by more than 25% as compared to that required for our previous PDMAPAAm-PNIPAM block copolymer.[23]

In this study, we used PDMAEMA as the surfactant to carry out deposition transfection. Similar to heparin immobilization, we thought that a small amount of surfactant would suffice for immobilizing DNA. We also hypothesized that the reduced amount of polymer would increase transgene expression. Therefore, we investigated the characteristics of PDMAEMA and its ability to combine with DNA to produce polyplexes. We then proceeded to optimize the deposition transfection conditions. Finally, we evaluated the effects of transfecting primary cells with PDMAEMA.

EXPERIMENTAL SECTION

N-(2-(Dimethylamino)ethyl)methacrylate (DMAEMA) and all other chemical reagents were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). DMAEMA was distilled under reduced pressure before use to remove the stabilizer.

General Methods. ^{1}H NMR spectra were recorded using a 300 MHz NMR spectrometer (Gemini 300; Varian, Palo Alto, CA) with deuterium oxide at room temperature. Gel permeation chromatography (GPC) analyses using *N,N*-dimethylformamide as a solvent were carried out by using an HPLC-8320 GPC instrument (Tosoh, Tokyo, Japan) in conjunction with Tosoh TSKgel SuperAW-4000 and SuperAW-5000 columns. The columns were calibrated prior to use with narrow distribution poly(ethylene glycol) standards (Tosoh).

Synthesis of Thermoresponsive Cationic Homopolymer. PDMAEMA was synthesized by photoradical polymerization. DMAEMA (2.0 g) was poured into a glass tube (35 × 65 mm^2; wall thickness, 1 mm, Maruemu Corp., Osaka, Japan), and N_2 gas was bubbled into it at 2 L/min for 10 min to remove O_2. DMAEMA was irradiated for 72 h using 100 mW visible light (FML27 EX-N; Panasonic Co., Osaka, Japan). After irradiation, reprecipitation was carried out using methanol solution in hexanes, and the precipitate was dried under reduced pressure, following which PDMAEMA was obtained (383 mg, 19% conversion). The molecular weight (M_n) of PDMAEMA was determined to be 2.5 × 10^5 g mol^{-1} (polydispersity: 2.5) by GPC analysis. Analysis of the ^{1}H NMR (in chloroform-d_1) spectra yielded the following results: δ 0.8–1.2 ppm (br, 3H, $-CH_3$), 1.6–2.0 (br, 2H, $-CH_2-CH_3$), 2.2–2.4 (br, 6H, $N-CH_3$), 2.5–2.7 (br, 2H, CH_2-N), 4.0–4.2 (br, 2H, $O-CH_2$). The mean diameters of PDMAEMA in deionized water (concentration: 10 mg/mL) were determined by dynamic light scattering (DLS) on Zetasizer Nano S (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 10 mW He–Ne laser (n = 5).

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Preparation of Polyplexes. PDMAEMA was dissolved in saline. Aliquots (60 μL) were added to the firefly luciferase-encoding plasmid DNA (pGL3 control vector; Promega, WI, USA) or green fluorescent protein-encoding plasmid DNA (pQBI25 vector; Wako) dissolved in 90 μL of DNase-free water (Invitrogen, CA, USA) to obtain polymer/pDNA ratios from 1 to 32, which corresponded to cation/anion (C/A) ratios. The solutions (total volume, 150 μL) were mixed using a pipet to generate DNA complexes. The mean diameters of the DNA complexes in deionized water (concentration: 10 mg/mL, C/A ratio: 4) were determined by dynamic light scattering (DLS) on Zetasizer Nano S (n = 5).

Surface Characterization. The chemical composition of the outermost surface layer was determined by X-ray photoelectron spectroscopy (XPS 3400; Shimazu Co., Kyoto, Japan) using a magnesium anode (Mg Kα radiation) at room temperature, a pressure of 5×10^{-6} Torr (10 kV, 20 mA), and a takeoff angle of 90°. The takeoff angle was defined as the angle between the sample surface and the electron optics of the energy analyzer.

Primary Cell Culture. Adipose-derived adherent cell populations were isolated from beagle adipose tissue by modifying a method described previously by Manini et al.[24] Briefly, approximately 1 g of adipose tissue was obtained from the fatty layer under the skin and digested using 0.1% collagenase type I solution (Wako) at 37 °C for 1 h with gentle agitation. After filtering the digest through a 100 μm nylon mesh (BD Biosciences, NJ, USA) and centrifuging it at 1300 rpm for 3 min, the cell pellet was collected. The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Corp., Carlsbad, CA) containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), penicillin (200 units/mL; ICN Biomedicals Inc., Aurora, OH), and streptomycin (200 mg/mL; ICN) (growth medium). Then, it was filtered through a 70 μm nylon mesh (BD Biosciences). The cells were placed on a dish (55 cm^2; Asahi Glass Co., Ltd., Tokyo) with the growth medium and cultured in an atmosphere of 5% CO_2 at 37 °C. When the culture was nearly confluent, it was harvested and subcultivated at 1.0×10^4 cells/cm^2. The cells were used for experiments before they reached the fifth passage.

In Vitro Transfection Assays. In vitro transfection by the deposition transfection method was performed as reported previously.[15] Briefly, an aqueous solution of the DNA complexes (50 μL; plasmid concentration, 20 μg/mL) was diluted with 150 μL of saline and then added into each well of a 24-well dish (amount of DNA added to each well, 1.0 μg). After incubation at 37 °C for 6 h, HeLa cells or adipose-derived primary cells (approximately 1.5×10^5 cells per well) in 1.5 mL of growth medium were seeded and cultured in an atmosphere of 5% CO_2 at 37 °C for 2–15 days, with the medium being changed every 3 days. The expression of pGL3-control-plasmid transfected cells was analyzed by a luciferase assay as follows. After 2–15 days of cultivation, the cells were lysed with 0.2 mL of cell lysis buffer (Promega). The lysate was centrifuged at 15 000 rpm for 3 min at 4 °C, and 20 μL of the supernatant was analyzed for luciferase activity using a Luminus CT-9000 luminometer (Dia-Iatron, Tokyo, Japan). The relative light unit (RLU) measurements were standardized by using the total protein amounts of the cell lysates, which were measured by performing BioRad protein assays (BIO-RAD, Hercules, CA) using bovine serum albumin as a standard. The GFP expression of pQBI25 transfected cells (transfection efficiency) was analyzed by using a fluorescence microscope fitted with a fluoroisothiocyanate filter.

In vitro transfection by the conventional solution transfection method was performed under optimized conditions as follows. HeLa cells (approximately 1.5×10^5 cells/well) were seeded prior to treatment in a 24-well dish and cultivated for 24–48 h in the growth medium in an atmosphere of 5% CO_2 at 37 °C. After 15 min of complex formation, transfection was performed by adding 100 μL of the complex solutions into each well of the 24-well dish with 0.5 mL of Opti-Mem I (Invitrogen, amount of DNA added to each well, 0.5 μg). After 6 h of incubation, the cells were washed once with PBS(−) and cultivated in 1.5 mL of the growth medium for 2–15 days. Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's procedures. Briefly, 2 μL of Lipofectamine 2000 reagent that was complexed with 0.5 μg of pDNA was added to each well. The luciferase and GFP expression were analyzed as described above.

Cell Viability Assays. Cytotoxicity was assessed by performing a cell viability assay using the water-soluble tetrazolium (WST)-8 method (Dojindo, Kumamoto, Japan). Polyplex solutions at different C/A ratios from 1 to 32 were added to each well in a 96-well plate (Asahi Glass Co. Ltd.). After incubating at 37 °C for 6 h, HeLa cells (approximately 1.5×10^4 cells per well) in 100 μL of the growth medium were seeded and cultured for 24 h at 37 °C in a 5% CO_2 atmosphere. To each well, 10 μL of WST-8 reagent (5 mmol/L) was added. After 2 h incubation at 37 °C, the absorbance at 450 nm was determined using a BioRad microplate reader (model 680).

RESULTS

Synthesis of PDMAEMA. PDMAEMA was synthesized by photoinduced radical polymerization by irradiating the bulk DMEMA using visible light. The resulting PDMAEMA was a solid white mass with a molecular weight of 2.5×10^5 g mol^{-1} and relatively narrow polydispersity. We were able to verify the chemical structure of the polymer by ^1H NMR spectroscopy (see Experimental Section for chemical shifts).

Characterization of DNA Complexes. The PDMAEMA thus obtained was dissolved in water at room temperature. The diameter of the polymer particles was 96 ± 3 nm. The polymer precipitated at approximately 28 °C, confirming its thermoresponsive nature (Figure 2). When an aqueous solution of PDMAEMA was mixed with an aqueous solution of the luciferase-encoding plasmid DNA (pGL3 control plasmid), the particle size increased by more than seven times (745 ± 46 nm), indicating the formation of polymer/pDNA complexes (polyplexes). The polyplexes precipitated (equilibrium transmittance, 15%; LCST, 30 °C) (Figure 2), which implies that PDMAEMA was sensitive to temperature even after polyplex formation.

Polyplex deposition was analyzed by XPS. Polystyrene (PS) was used as a model material. It was coated with PDMAEMA in saline or as a complex with pGL3 plasmid, subsequently air-dried, and then washed with water for 24 h at 37 °C. After coating, an N_{1s} signal, which originated mainly from the dimethylamino groups in the side chains of PDMAEMA, was detected on the surface. The changes in chemical composition are summarized in Table 1. For the polymer coating, the N/C elemental ratio determined from the peak area of C_{1s} and N_{1s} was 0.08 before and after washing with water, which was close to the theoretical ratio of PDMAEMA (0.12). On the other hand, when the polyplexes were coated on to the PS surface, a

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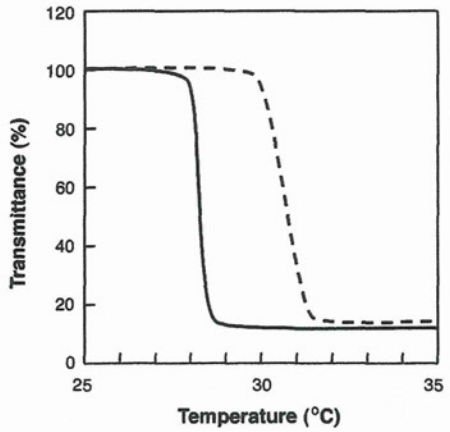

Figure 2. Thermoresponsive changes in transmittance of PDMAEMA (solid line) and its polyplex with pGL3 control plasmid DNA (broken line). Polymer and its polyplex (C/A ratio: 4) concentration: 10 mg/mL, Heating rate: 0.5 °C/min.

Table 1. Change in Surface Chemical Composition of the PS Film before and after Coating with PDMAEMA and its Polyplex with pGL3 Control Plasmid[a]

surface	chemical ratio N/C	chemical ratio P/C
PS	0 (0)	0 (0)
coating with PDMAEMA	0.08 (0.12)	0 (0)
washing at 37 °C for 24 h	0.08 (0.12)	0 (0)
coating with polyplex	0.13 (0.26)	0.02 (0.05)
washing at 37 °C for 24 h	0.11 (0.26)	0.02 (0.05)

[a]Theoretical ratios are shown in parentheses. The data are presented as means (n = 5).

new P_{1s} signal was detected that mainly originated from the phosphate group in addition to the N_{1s} group. The N/C and P/C ratios were 0.13 and 0.02, respectively. Each ratio remained nearly stable even after washing for 24 h. Therefore, the polyplexes of PDMAEMA with pDNA could be immobilized thermally on the PS surface with high durability at 37 °C.

In Vitro Transfection and Cell Viability. HeLa cells were transfected by the deposition transfection method and the conventional transfection method. Polyplexes of PDMAEMA and the luciferase-encoding pGL3 control plasmid were created with C/A ratios ranging from 1 to 32. The luciferase activity was estimated through the levels of transgene expression 2–15 days post-transfection. When the cells were transfected by the conventional method, relatively high transgene expression levels were detected over the C/A ratio range 8–16. On the other hand, significantly high transgene expressions were observed at low C/A ratios of 2 and 4 for the deposition method, which were higher than the highest transgene expression C/A ratio of 32 obtained for the conventional method (Figure 3).

The high transgene expression of the deposition method was maintained for over 2 weeks, whereas the transgene expression of the conventional method decreased dramatically after 2 days. Fifteen days post-transfection, the transgene gene expression activity resulting from the deposition method was more than 100 times that resulting from the conventional method (Figure 4A). The number of GFP positive cells as a transfection

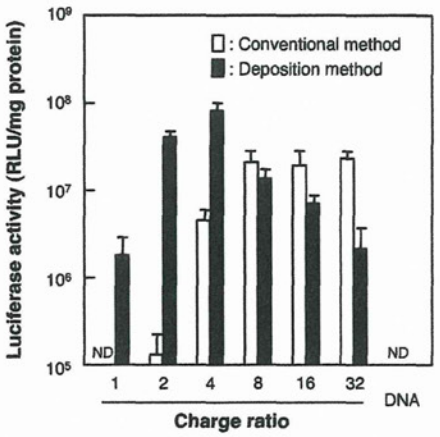

Figure 3. Effect of C/A ratio on transgene expression levels of HeLa cells transfected by using conventional method and deposition method with PDMAEMA. The luciferase activities of the conventional and the deposition method were measured at 2 and 5 days of post-transfection, respectively. The values represent the mean ± SD (n = 3). DNA indicates transfection without polymer. ND: not detected.

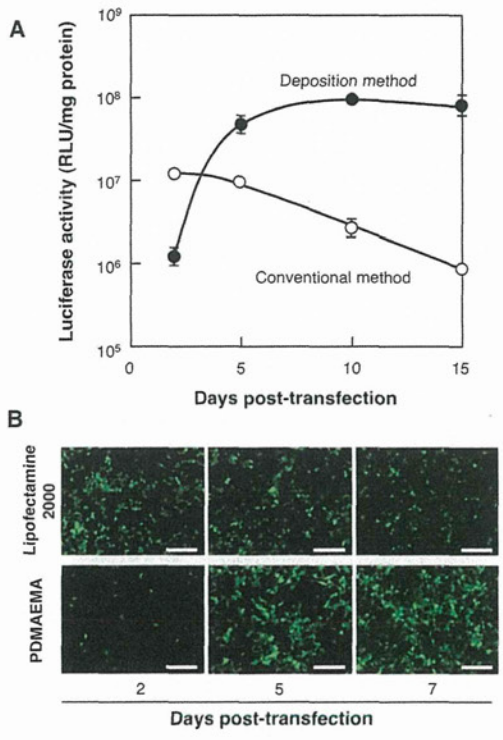

Figure 4. (A) Time course of the luciferase expression levels of HeLa cells transfected by conventional transfection method (○) and deposition transfection method (●) using polyplexes of PDMAEMA with pGL3-control plasmid DNA having C/A ratio of 16 and 4, respectively. The values represent the mean ± SD (n = 3). (B) Fluorescence microscopy observations of the HeLa cells transfected with pQBI25 plasmid DNA by conventional transfection method using Lipofectamine 2000 and deposition transfection method using PDMAEMA having C/A ratio of 4. Bar = 200 μm.

efficiency of deposition transfection method using PDMAEMA was significantly increased between 2 and 5 days of post-

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transfection (Figure 4B), which was the same tendency of the time course of transgene expression level of the deposition method shown in Figure 4A. Moreover, the transfection efficiency obtained by the deposition transfection using PDMAEMA was the same as or higher than that obtained by the conventional transfection using commercially available Lipofectamine 2000 at 5 days post-transfection (Figure 4B).

The viability of the HeLa cells transfected by employing the deposition method using PDMAEMA was evaluated for C/A ratios ranging from 1 to 32. Although the cell viability decreased slightly with increasing C/A ratio, significantly high cell viability (more than 90%) was obtained for C/A ratios ranging from 2 to 8, which were higher than those obtained by using commercially available Lipofectamine 2000 (Figure 5).

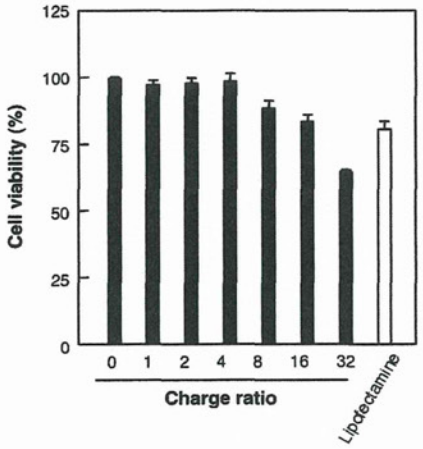

Figure 5. Cytotoxicity of polyplexes prepared by mixing PDMAEMA and pGL3 control plasmid DNA in different charge ratios (black bars) and lipoplexes prepared by mixing Lipofectamine 2000 and pGL3 control plasmid (white bar). The values represent the mean ± SD (n = 3).

Notably, a C/A ratio of 4, which showed the highest transgene expression for the deposition transfection method, also resulted in the highest cell viability (97%). This result was almost identical to that for the nontreated cells.

To evaluate the applicability of PDMAEMA to gene therapy, a preliminary study of in vitro transfection using adipose-derived primary cells was conducted. Adipose-derived primary cells were separated from beagle adipose tissue and transfected by using the deposition method and pQBI25 plasmid. As shown in Figure 6, the GFP positive primary cell populations were clearly visible in the fluorescence microscope images.

DISCUSSION

In a previous study, we developed the substrate-mediated deposition transfection method by using a custom-designed, tertiary-branched thermoresponsive cationic block copolymer (tertiary branched PDMAPAAm-PNIPAM block copolymer).[15] PDMAPAAm had cationic blocks for forming polyplexes with DNA and PNIPAM blocks for thermoresponsive surface immobilization of the polyplexes. The long chains of the PNIPAM blocks were effective for surface immobilization, but they reduced transgene expression because of DNA coverage by the polymer. On the other hand, the long chains of the PDMAPAAm blocks enabled firm binding of the polymer with DNA, but the deposited polyplex layer was delaminated easily

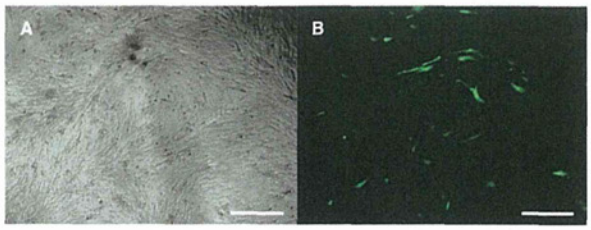

Figure 6. Fluorescence microscopy observations of adipose-derived primary cells transfected by using deposition method with polyplexes of PDMAEMA and pQBI25 plasmid DNA at C/A ratio of 4. Microscopic observation: 5 days post-transfection. (A) phase contrast microscopic image and (B) fluorescence microscopic image. Bar = 100 μm.

by washing. Therefore, a balance between the thermoresponsive and cationic blocks was needed for effective transgene expression by deposition transfection. We found through optimization that the chain length of PNIPAM should be twice as long as the PDMAPAAm chain length.

In this study, we attempted to use PDMAEMA, which has both thermoresponsive and cationic characteristics, for deposition transfection. As expected, PDMAEMA formed polyplexes with DNA and they could be immobilized on the substrate surface with high durability. Therefore, we could obtain high transgene expression using the deposition transfection method in conjunction with low amounts of PDMAEMA. For example, PDMAEMA was mixed with the same amount of pDNA to form polyplexes at a C/A ratio of 4. In comparison, for the previous tertiary branched PDMAPAAm-PNIPAM block copolymer, a 3-fold excess of the polymer relative to the pDNA was necessary to form polyplexes at the same C/A ratio.[15] Interestingly, significantly high cell viabilities (more than 90%) were obtained when the cells were cultured on the PDMAEMA/pDNA polyplex culture surface over C/A ratios ranging from 2 to 8 (Figure 5). This might have been due to the effect on cell growth of reducing the use of the polymer.

Positively charged polyplexes prepared at high C/A ratios performed better in terms of transfecting cells because of their enhanced ability to bind to negatively charged proteoglycans on cell surfaces.[25] Indeed, a high transfection efficiency was achieved by using the conventional solution-mediated transfection method with polyplexes of PDMAEMA at high C/A ratios ranging from 15 to 30.[16,26] These results agree well with the results that we obtained by using the conventional transfection method at a C/A ratio of 32 (Figure 3). On the other hand, the highest level of transgene expression that we achieved in this study occurred at a C/A ratio of 4 for the deposition transfection method. The reason this occurred is unclear. In our previous study, when the polyplexes were formed from tertiary branched PDMAPAAm-PNIPAM block copolymers, they were fixed firmly to the culture substrate by drying (reverse transfection method). In addition, the transgene expression level was decreased by more than four times as compared to deposition transfection where the polyplexes were attached weakly to the culture substrate.[15] Therefore, the effect of the C/A ratio on transgene expression that we observed in this study may have been due to the polyplexes being prepared by mixing pDNA with high amounts of PDMAEMA (C/A = 16, 32). Through this, the polyplexes may have more strongly attached to the culture surface than those that were prepared by

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mixing pDNA with low amounts of PDMAEMA (C/A = 2, 4). Adsorption occurred likely through hydrophobic interactions between the PDMAEMA and hydrophobic cell culture surface. As a result, if the polyplexes with high C/A ratios were too tightly bound to the surface, their uptake by cells may have been inhibited.[15] A detailed analysis of the relationship between the C/A ratios of the polyplexes and cellular uptake via the deposition transfection method is required. The results derived from such a study would be helpful for clinical applications of PDMAEMA with the aim of achieving high transgene expression using the deposition transfection method in conjunction with low amounts of PDMAEMA.

In general, strong transgene expression is achieved within the first 2−5 days post-transfection using the conventional transfection method. However, expression decreases dramatically 1 week post-transfection.[27] This observation is in agreement with the time course evaluation of transgene expression that we carried out for the conventional transfection method in this study (Figure 4A). On the other hand, sustained transgene expression even after 2 weeks post-transfection was observed by using cells that had been transfected through deposition transfection (Figure 4A). Short-term transgene expression achieved using the conventional transfection method is thought to be due to mainly two rate-limiting factors. First, polyplexes cannot be supplied continuously to the cells. It is necessary to exchange the serum-free medium with serum containing growth medium to foster cell growth. Any polyplexes that would be present in the new medium would lose their transfection activity because they would interact with the serum components. Second, for nonviral, plasmid-mediated transfection, incorporating a transgene into the genome of a host cell is extremely difficult.[28] In this study, the first rate-limiting factor did not exist for the deposition transfection method because the polyplexes were deposited and adsorbed on to the culture surface and they were kept in contact with the culture surface even after washing and the medium exchange as shown in Table 1. Therefore, the polyplexes were supplied continuously to the cells from the culture surface, and as a result, sustained transgene expression was achieved. Indeed, Okazaki et al. also reported that transgene expression was sustained at least 8 days post-transfection for the substrate-mediated transfection method using cationic pullulan, whereas transgene expression was decreased significantly just 4 days post-transfection for the conventional transfection method.[29]

Although the transgene expression level and transfection efficiency at 2 days of post-transfection in the deposition method were relatively low compared to those in the conventional method, they were significantly increased between 2 and 5 days of post-transfection (Figure 4A,B). The conventional solution-mediated transfection was performed by adding polyplexes to log growth-phase cells, which have high activity against polyplex uptake through cell endocyosis. On the other hand, deposition transfection was performed by seeding cells on the polyplex deposited culture surface, in which the activity of polyplex uptake by the cells would be very low until cells reach log growth phase, because they initiate uptake of polyplexes and other molecules after their adhesion to culture surface and then log-phase growth. Therefore, we considered that the difference in the time course of transgene expression profile between conventional and deposition transfection method was caused by the difference in cell state when the cells were exposed to polyplexes.

In the conventional transfection method, cells are needed to precultivate for several days before transfection to obtain growth-phase cells. On the other hand, in the deposition method, cells are directly seeded on the polyplex-deposited culture well, and they are transfected according with their growth. So, the time to obtain transgene expression from the starting point of cell cultivation is not so different between conventional and deposition methods. Therefore, we consider that the delay of transgene expression in the deposition transfection is not a disadvantage for the use of cell culture applications.

As shown in Figure 6, we successfully transfected the GFP gene into the primary cells isolated from beagle adipose tissue on the polyplex culture surface. Stem-cell-mediated tissue engineering and gene therapy are promising approaches for many areas of tissue regenerative medicine.[30] In particular, adipose-derived adherent cell populations including adipose-derived stem cells are useful for those approaches because they can differentiate into mesodermal tissues including adipocytes, chondrocytes, smooth muscle cells, and cardiac cells. Moreover, they are noninvasive and can be easily isolated from the human body.[31,32] Therefore, our results for transfecting adipose-derived primary cells by utilizing deposition transfection mean that deposition transfection using PDMAEMA may be a potential tool for stem-cell-mediated tissue engineering and gene therapy.

■ CONCLUSION

By using small amounts of PDMAEMA with both thermoresponsive and cationic characteristics, high and sustained transgene expression was achieved via deposition transfection, thus resulting in high cell viability. Further, the PDMAEMA-mediated transfection of adipose-derived primary cells in vitro was highly efficient. We believe that our findings confirm the applicability of PDMAEMA as a deposition transfection material and as a valuable tool for gene therapy.

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Notes

The authors declare no competing financial interest.

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Observation of local elastic distribution in aortic tissues under static strain condition by use of a scanning haptic microscope

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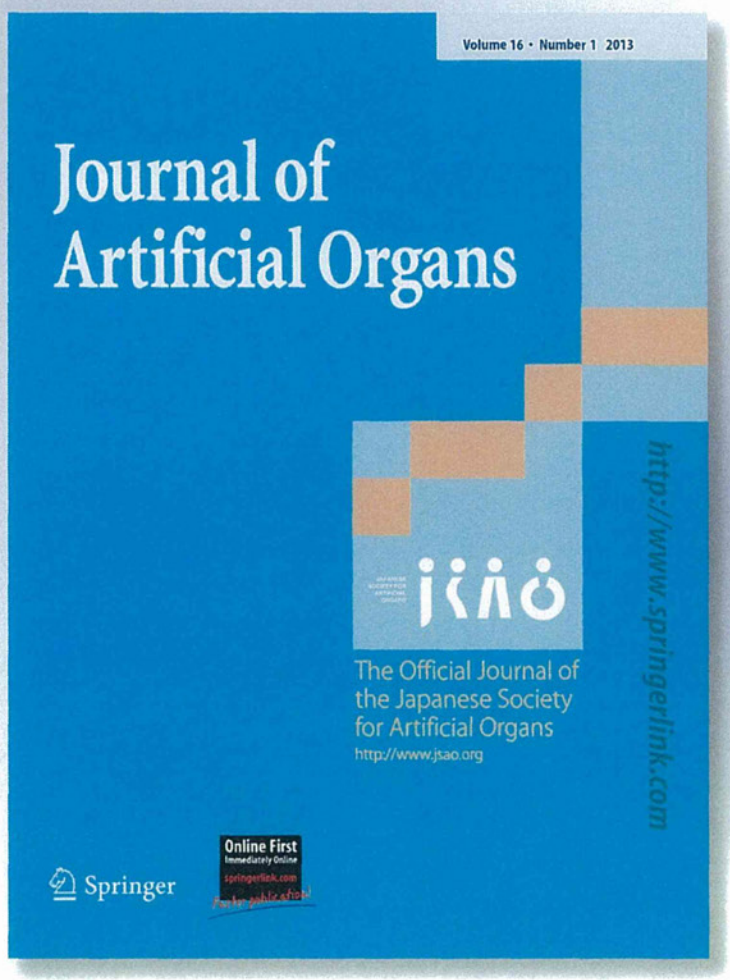

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ORIGINAL ARTICLE

Observation of local elastic distribution in aortic tissues under static strain condition by use of a scanning haptic microscope

Takeshi Moriwaki · Tomonori Oie · Keiichi Takamizawa · Yoshinobu Murayama · Toru Fukuda · Sadao Omata · Yasuhide Nakayama

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Abstract The purpose of this study was to observe variation in the local elastic distribution in aortic tissue walls under different static strain conditions, including physiological strain, by use of a scanning haptic microscope (SHM). Strain was applied by stretching aortic tissues in the circumferential direction by the simple tensile method or by the rod-insertion method to mimic in vivo internal pressure loading. SHM measurements in a saline solution at room temperature were performed on canine thoracic aorta using a glass needle probe with a diameter of ca 5 μm and a scanning area and point pitch of 160 × 80 μm and 2 μm, respectively. Under strain of 0–0.23, corresponding to internal pressure of 0–150 mmHg, wavy-shaped elastin fibers stretched until they were almost straightened, and the average elastic modulus increased almost linearly. Although there was little difference between the images obtained for the two different stretching methods, under high strain (>0.36; 250 mmHg) significant circumferential orientation of the collagen fibrils occurred with an increase in the average elastic modulus. It was concluded that the pressure resistance of the aorta under physiological strain was mainly afforded by elastin fibers; collagen fibrils contributed little except under much higher pressures.

Keywords Scanning haptic microscope · Elastic distribution · Aortic tissue · Strain

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Introduction

The aorta, the largest artery in the body, carries oxygenated blood to all parts of the body. It acts both as a conduit and as an elastic chamber for pulsatile blood flow. Its elasticity serves to convert the heart's pulsatile flow into a steady flow. Aortic function is closely related to its mechanical properties. Thus far, the mechanical properties of the aorta have been evaluated by use of a tensile test [1–3] and an internal pressure load test [4–6]. Although the former is quite a simple test, a disadvantage is that data are obtained by nonphysiological uniaxial extension of the aorta. In contrast, in the latter test data are obtained by physiological extension. Unfortunately, in both tests, the mechanical properties measured are average features through the aortic wall. The aorta consists of three layers—intima, media, and adventitia—that consist of cells and extracellular matrices. The matrices are mainly composed of elastin fibers or collagen fibrils, which contribute to the mechanical properties of the aorta. The abovementioned tests cannot be used to evaluate the microscopic distribution of the mechanical properties of aortic tissue. Therefore, a combination of a macroscopic mechanical test and theoretical analysis has been used to estimate the mechanical properties of aortic tissue [7, 8].

Recently, many studies have attempted to evaluate the local mechanical properties of aortic tissue. Atomic force microscopy (AFM) is popularly used for this purpose,